Identification of connexin 50 and 57 mRNA in A-type horizontal cells of the rabbit retina

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ABSTRACT

Horizontal cells (HCs) mediate negative feedback to photoreceptors. In the mammalian retina, there are two types of HCs, which are extensively coupled to neighboring cells through homologous gap junctions. The permeability and therefore the strength of feedback can be regulated by light intensity, dopamine and many other factors. However, the component(s) of the most prominent gap junctions, those between A-type HCs in the rabbit retina, is still unknown. In this study, we compared the sequences of many types of mammalian connexins, obtained partial sequences of rabbit connexin 50 and 57. Using specific primers designed against the rabbit sequences, we identified mRNAs of connexin 50 and/or 57 in visually selected single A-type HC using multiplex RT-PCR.

Keywords: gap junctions, dissociated retinal neurons, single cell multiplex RT-PCR, sequencing.

INTRODUCTION

In the central nervous system, neurons communicate with each other through two types of synapses, chemical (conventional) synapses and electrical synapses. The electrical synapses, also known as gap junctions are small pores across neighboring membranes formed by connexins (Cxs). In the retina, intracellular injection of Neurobiotin showed that gap junctions exist among same type of neurons (homologous coupling) or among different types of neurons (heterologous coupling) [1]. Since the identification of the first neuronal connexin, Cx35 in the fish retina [2], a number of connexins have been identified [3]. The functions of some connexins have been well studied, see reviews [4, 5]. For example, Cx36, the murine ortholog of fish Cx35, has been shown to locate in the rod pathway and to play an essential role in the transmission of rod signals [6-10]. Cx26 has been shown to form hemichannels on horizontal cells (HCs) and has been proposed to play a role in feedback mechanism to cones [11].

Electron microscopy studies demonstrated the existence of gap junctions between A-type HCs decades ago [12-14]. Electrophysiological recordings supported the notion by showing that the receptive fields of A-type HCs exceed their dendritic fields for many folds in size [15]. Lucifer Yellow could readily pass through the gap junctions. Using the extent of coupling as a measure, it has been revealed that the permeability of this gap junctions is regulated by dopamine, pH, retinoic acid and intracellular cAMP [16, 17], see reviews [18, 19]. However, the knowledge about component(s) of this most prominent gap junction in the retina is still limited. Existing evidence suggested that it was neither Cx26 or Cx36 [20]. There are suggestions that Cx50 may form gap junctions between A-type HCs in rabbit retina, and Cx57 between HCs in the mouse retina [21, 22]. However, mouse has been shown to only possess one type, axon-bearing HCs [23, 24]. In this study, we obtained partial sequences of rabbit Cx50 and 57, identified mRNAs of Cx50 and 57 in visually selected, dissociated single A-type HCs of rabbit retina using multiplex RT-PCR.

MATERIALS AND METHODS

All procedures of handling experimental animals have been approved by the institutional committee and conform to the NIH guide for the Care and Use of Laboratory Animals. Adult New Zealand White rabbits were overdosed with a mixture of Ketamine and

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Xylazine. Eyes were quickly enucleated and retinas were carefully isolated in oxygenated Ames solution for later use. In the initial phase, 10 µl 4',6'-diamidino-2-phenylindole (DAPI 1 µg/µl, Sigma) were injected intraocularly under anesthesia with Ketamine and Xylazine one day before experiment, in order to distinguish A-type HCs from axon terminals of B-type HCs.

The methods of obtaining isolated A-type HCs have been described in detail previously [25]. The retina was dissociated by mild mechanical trituration after treated in a mixture of 80 U/ml papain

(Sigma) and 0.1 mg/ml L-cysteine (Sangon, Shanghai) in Earle's balanced salt solution (EBSS, Sigma) at 37°C for 15 min. The suspension of cells was diluted in HEPES buffer (NaCl 135 mM, KCl 5 mM, MgCl₂ 1 mM, CaCl₂ 2 mM, HEPES 10 mM, Glucose 10 mM, BSA 0.1 mg/ml, pH7.4). A-type HCs were easily identified by their unique morphology using a Nikon E600FN microscope equipped with a 10 × objective (Fluor N.A. 0.3). A glass pipette with a tip of approximately 10 µM, filled with EBSS containing Yeast tRNA carrier (1 µg/µl, 15401-011, Gibco, Grand Island, NY), RNase inhibitor

Fig. 1 Design and refinement of nucleotide sequence specific primers for rabbit Cx50 and 57. Amino acid sequence of 13 human connexins, mouse Cx57 and zebra fish Cx55.5 are analyzed using multiple alignment. Different amino acids in different species are darkened. For clarity, only nine human connexin sequences and the mouse Cx57 sequence are shown. An upstream amino acid sequence specific for Cx57 (57du1) and a downstream sequence common to all connexins (dd1) were used to design the degenerative primers (annotated above the arrows). The primers used in the 2nd round PCR are marked as 57du2 and 57d2. A pair of primers marked by 57u3 and 57d3 is designed using nucleotide sequences specific for rabbit Cx57, and used in the single cell PCR. The design and refinement of specific primers for rabbit Cx50 is similar and marked with 50du1, dd1, 50u2 and 50d2.

(0.5 U/ μ l, SNBC, Shanghai) was used to translocate the HCs to 0.2 ml PCR tubes containing 1 μ l H₂O with 1 μ g/ μ l Yeast tRNA and 0.5 U/ μ l RNase inhibitor. The tube was quickly stored in liquid nitrogen for later use.

If not specified, the first strand cDNA of the whole retina or of single A-type HCs was synthesized using Superscript First-Strand Synthesis System for RT-PCR (11904-018, Gibco) according to manufacturer's instructions.

For the PCR amplification, 1 μ l diluted RT product was added into the mixture containing following components: 2.5 μ l 10 $\times$ PCR-buffer (SNBC, Shanghai); 1.5 μ l 25 mM MgCl₂ (SNBC, Shanghai); 0.5 μ l 10 mM dNTP (TaKaRa); 0.5 μ l 50 μ M of each downstream and upstream degenerative primers (SNBC, Shanghai); 0.25 μ l Taq polymerase (5 U/ μ l, SNBC, Shanghai) and 18.5 μ l DEPC-H₂O (Sangon, Shanghai). A hot-start PCR was performed by directly incubating the PCR tube at 95°C, followed by a PCR program: 95°C for 3 min, 40 cycles of 94°C for 30 sec, 59°C for 30 sec (the annealing temperature decreases 0.5°C every cycle until it reaches 54°C), 72°C for 60 sec followed by a 15 min extension at 72°C. The PCR products were loaded into 1.8% agarose gel and the expected band was purified by the Gel (PCR) Purification Kit (Qiagen), cloned into the pUCMT (SNBC, Shanghai) and sequenced.

For single cell RT-PCR, selected HC was digested with RNase-free DNaseI (TaKaRa) and mRNA was reverse transcribed into cDNA as described above. The cDNA was amplified in the reaction mixture composed of 30 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.0 mM MgCl₂, 0.08% NP40, 200 μ M each dNTP, 1.0 unit Taq polymerase and 200 nM each of nucleotide sequence specific primers for GAPDH (designed according to the Genebank L23961), or Cx50 or Cx57 (see results and Fig. 1), executing a series of PCR reactions: 95°C for 3 min, 40 cycles of (94°C for 30 sec, 55°C for 30 sec, 72°C for 45 sec) and 72°C for 7 min.

For single cell multiplex RT-PCR, the procedures were almost identical to single cell RT-PCR. The only difference is the nucleotide sequence specific primers for Cx57, Cx50 and GAPDH were introduced into the PCR reaction mixture at the same time. Products (15 μ l) of the first round PCR were purified with the PCR purification kit (SNBC, Shanghai). Purified aliquots (3 μ l) were amplified separately in a second-round, 35-cycle PCR reaction using sequence specific primers of Cx57, Cx50 and GAPDH, respectively.

RESULTS AND DISCUSSION

We analyzed thirteen human connexin proteins (Cx25, 26, 30, 31, 32, 36, 37, 40, 43, 45, 50, 58, 62), zebra fish Cx55.5 and mouse Cx57 by multiple sequence alignment (Fig. 1). We designed an upstream degenerative primer using an amino acid sequence specific for homologs of Cx57 (AQMENPE) (57du1: 5'-GCYCAGATGGARAAAYCC HGA3') and a downstream degenerative primer using an amino acid sequence derived from a conserved region of all fifteen connexins (PTEKTVF) (dd1: 5'-GAAGACGGT YTTYTCKGTRGG3') (Fig. 1). A 285 bp band derived from whole retina cDNA was purified, cloned and sequenced. The amino acid sequence revealed a fragment with 88% homology to mouse Cx57 (Genebank NP_034419) and 95% homology to human Cx62 (Genebank NP_115991).

To further improve the specificity of the primers for

rabbit Cx57, we used the whole retina cDNA for another round of PCR using an upstream degenerative primer against an amino acid sequence specific for homologs of Cx57 (57du2: 5'-ATGGGSGACTGGAAYCTSYT3') (Fig. 1) and a downstream primer against a nucleotide sequence specific for rabbit Cx57 (57d2: 5'-GGGACAAGGA GGCTGAGTGCATTT3') (Fig. 1). Using these two primers, we obtained a 582 bp band. When this band was cloned, sequenced and combined with the result of previous experiment, a partial gene sequence of rabbit Cx57 is obtained (Genebank accession number AY515298) (Fig. 1). The amino acid sequence of the partial rabbit Cx57 shows a homology of 92% to mouse Cx57 (Genebank NP_034419) and 94% homology to human Cx62 (Genebank NP_115991), respectively.

A partial sequence of rabbit Cx50 (Genebank accession number AY603359) is obtained by using the upstream degenerative primer against an amino acid sequence specific for homologs of Cx50 (CHIFFKT) (50du1: 5'-TGCCACAT YATYTTYAARAC3') and the downstream primer dd1 (Fig. 1). The amino acid sequence of this 159 bp fragment

Fig. 2 Identification and confirmation of A-type HCs. (A) morphologically identified A-type HCs among dissociated rabbit retinal neurons. (B) presence of a nucleus under UV illumination when stained with DAPI. Scale bar: 20 μ m.

showed 100% homology to mouse Cx50 (Genebank NP_032149) and human Cx50 (Genebank NP_005258).

A-type HCs have morphology similar to that of the axon terminals of B-type HCs. To rule out the possible contamination of axon terminals of B-type HCs, we stained the retina with DAPI overnight before dissociation. The identity of A-type HCs was confirmed by the presence of a nucleus (Fig. 2). All 80 A-type HCs identified by morphology had visible nuclei under UV illumination. Although we did not use any HCs exposed to UV as templates for single cell RT-PCR due to possible UV damage to nucleic acids, we are confident that a predominant proportion of cells selected are indeed A-type HCs.

A pair of upstream and downstream primers (57u3 and 57d3) against the nucleotide sequence specific for rabbit Cx57 were designed and used for single cell RT-PCR (Fig. 1). Using these primers, Cx57 mRNA has been identified in single and multiple (2-4) visually selected A-type HCs (qualitatively similar to Fig. 3). Similarly, using nucleotide sequence specific primers (50u2 and 50d2, Fig. 1), Cx50 mRNA has also been detected in single A-type HCs (qualitatively similar to Fig. 3).

Several controls were carried out. GAPDH was used as a positive control to make sure that mRNAs of the HCs were reverse transcribed and amplified. The whole retina cDNA was used as a positive control to check the size of the band obtained from single cell PCR (data not shown). Negative controls using H₂O, HEPES buffer taken from the dish as templates, treated with DNaseI and reverse transcriptase, and HCs treated with DNaseI but without reverse transcriptase were carried out to rule out possible contamination from PCR system, extracellular mRNA and genomic DNA.

Fig. 3 Single cell multiplex RT-PCR. The bands (GAPDH: 449 bp; Cx57: 340 bp; Cx50: 148 bp) in the same column show the 2nd round PCR products from the same HC. Lane 1: marker; 2: H₂O as template; 3: HC without reverse transcriptase; 4 and 5: extracellular fluid for cells 6-12; 6-12: HCs; 13: extracellular fluid for cell 14; 14: HC; 15: extracellular fluid for cells 16-18; 16-18: HCs.

To answer the question whether Cx50 and Cx57 are expressed in the same HCs, we performed single cell multiplex RT-PCR checking mRNAs for Cx50, Cx57 and GAPDH simultaneously. Out of 8 cells showed GAPDH positive band, two cells showed positive signal for Cx57, three cells for Cx50, one cell exhibited both Cx57 and Cx50 signals (Fig. 3).

In this study, we obtained partial gene sequences of rabbit Cx50 and 57, designed nucleotide sequence specific primers and identified mRNAs of Cx50 and Cx57 at the level of single visually identified A-type HCs. Our results indicate that these connexins could be utilized to form the most prominent gap junctions in the rabbit retina.

The gap junctions between A-type HCs have some unique properties. They have a large conductance reflected in the size of their receptive fields, which exceeds the dendritic fields several times. They are permeable to negatively charged Lucifer Yellow molecules whereas most gap junctions in the retina are only permeable to positively charged Neurobiotin. This may due to charge selectivity rather than pore size [26]. These properties lead us to target Cx50 and Cx57, homologs of fish Cx44.1 and Cx55.5. The heterotypic channels formed by Cx44.1 and Cx55.5 in functional expression system showed large conductance and strong rectification [3]. Indeed, our results showed that Cx50 and Cx57 are transcribed in the A-type HCs in the rabbit retina.

The relatively low success rate is likely due to the technically demanding single cell manipulation. Firstly, the acute dissociation may injury horizontal cells: the digestion with papain and subsequent dissociation by mechanical force may disrupt the dendrite of A-type HC. The cells may initiate apoptosis when it suffered this damage, therefore most mRNAs might be degraded. Secondly, although we made a lot of efforts to ensure that the selected cell did enter the pipette, and re-examined the pipette after expelling the cell into the PCR tube, it is hard to be certain that the cell is indeed present in the PCR tube. Thirdly, the minute amount of mRNA is easily degraded. Therefore, the GAPDH control is critical, an indication of the presence of the HC. In some cases, GAPDH could also be detected in extracellular solution (Fig. 3, Lane 13), probably due to the contamination of small cells when sampling extracellular solution, or due to high concentration of GAPDH mRNA in the solution during dissociation. The data were excluded if extracellular solution showing positive GAPDH.

The identification of mRNAs cannot be equated to be the expression of functional connexins. We attempt to examine the connexin protein expression by immunohistochemistry but failed to do so, because the antibody didn't work in the rabbit retina.

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REFERENCES

- 1 Vaney DI. Many diverse types of retinal neurons show tracer coupling when injected with biocytin or Neurobiotin. *Neurosci Lett* 1991; **125**:187-90.
- 2 O'Brien J, Bruzzone R, White TW, *et al*. Cloning and expression of two related connexins from the perch retina define a distinct subgroup of the connexin family. *J Neurosci* 1998; **18**:7625-37.
- 3 Dermietzel R, Kremer M, Paputsoglu G, *et al*. Molecular and functional diversity of neural connexins in the retina. *J Neurosci* 2000; **20**:8331-43.
- 4 He S, Dong W, Deng Q, *et al*. Seeing more clearly: recent advances in understanding retinal circuitry. *Science* 2003; **302**:408-11.
- 5 Sohl G, Guldenagel M, Traub O, Willecke K. Connexin expression in the retina. *Brain Res Brain Res Rev* 2000; **32**:138-45.
- 6 Feigenspan A, Teubner B, Willecke K, Weiler R. Expression of neuronal connexin36 in all amacrine cells of the mammalian retina. *J Neurosci* 2001; **21**:230-9.
- 7 Deans MR, Volgyi B, Goodenough DA, *et al*. Connexin36 is essential for transmission of rod-mediated visual signals in the mammalian retina. *Neuron* 2002; **36**:703-12.
- 8 Guldenagel M, Ammermuller J, Feigenspan A, *et al*. Visual transmission deficits in mice with targeted disruption of the gap junction gene connexin36. *J Neurosci* 2001; **21**:6036-44.
- 9 Mills SL, O'Brien JJ, Li W, *et al*. Rod pathways in the mammalian retina use connexin 36. *J Comp Neurol* 2001; **436**:336-50.
- 10 Sohl G, Degen J, Teubner B, Willecke K. The murine gap junction gene connexin36 is highly expressed in mouse retina and regulated during brain development. *FEBS Lett* 1998; **428**:27-31.
- 11 Kamermans M, Fahrenfort I, Schultz K, *et al*. Hemichannel-mediated inhibition in the outer retina. *Science* 2001; **292**:1178-80.
- 12 Fisher SK, Boycott BB. Synaptic connections made by horizontal cells within the outer plexiform layer of the retina of the cat and the rabbit. *Proc R Soc Lond B Biol Sci* 1974; **186**:317-31.
- 13 Raviola E, Gilula NB. Intramembrane organization of specialized contacts in the outer plexiform layer of the retina. A freeze-fracture study in monkeys and rabbits. *J Cell Biol* 1975; **65**:192-222.
- 14 Kolb H. The organization of the outer plexiform layer in the retina of the cat: electron microscopic observations. *J Neurocytol* 1977; **6**:131-53.
- 15 Bloomfield SA, Miller RF. A physiological and morphological study of the horizontal cell types of the rabbit retina. *J Comp Neurol* 1982; **208**:288-303.
- 16 Hampson EC, Weiler R, Vaney DI. pH-gated dopaminergic modulation of horizontal cell gap junctions in mammalian retina. *Proc R Soc Lond B Biol Sci* 1994; **255**:67-72.
- 17 Weiler R, He S, Vaney DI. Retinoic acid modulates gap junctional permeability between horizontal cells of the mammalian retina. *Eur J Neurosci* 1999; **11**:3346-50.
- 18 Baldrige WH, Vaney DI, Weiler R. The modulation of intercellular coupling in the retina. *Semin Cell Dev Biol* 1998; **9**:311-8.
- 19 Weiler R, Pottek M, He S, Vaney DI. Modulation of coupling between retinal horizontal cells by retinoic acid and endogenous dopamine. *Brain Res Brain Res Rev* 2000; **32**:121-9.
- 20 Deans MR, Paul DL. Mouse horizontal cells do not express connexin26 or connexin36. *Cell Commun Adhes* 2001; **8**:361-6.
- 21 Hombach S, Janssen-Bienhold U, Sohl G, *et al*. Functional expression of connexin57 in horizontal cells of the mouse retina. *Eur J Neurosci* 2004; **19**:2633-40.
- 22 Massey SC, O'Brien JJ, Trexler EB, *et al*. Multiple neuronal connexins in the Mammalian retina. *Cell Commun Adhes* 2003; **10**:425-30.
- 23 He S, Weiler R, Vaney DI. Endogenous dopaminergic regulation of horizontal cell coupling in the mammalian retina. *J Comp Neurol* 2000; **418**:33-40.
- 24 Peichl L, Gonzalez-Soriano J. Unexpected presence of neurofilaments in axon-bearing horizontal cells of the mammalian retina. *J Neurosci* 1993; **13**:4091-100.
- 25 Li H, Gaughwin P, Li N, He S. Localization of dopamine D1-receptor to A-type horizontal cells in the rabbit retina by single cell RT-PCR. *Neurosci Lett* 2004; **355**:146-8.
- 26 Mills SL, Massey SC. A series of biotinylated tracers distinguishes three types of gap junction in retina. *J Neurosci* 2000; **20**:8629-36.